# Expression of Apoptosis Regulatory Proteins in the Skeletal Muscle of Tumor-bearing Rabbits

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**We reported finding that apoptosis occurred in skeletal muscle in the early stage after implantation. In the present study, we investigated expression of the apoptosis-related proteins Bax and Bcl-2 to determine the mechanism of the apoptosis. In the early stage of tumor bearing, 20 days after implantation, lean body mass (LBM) was reduced by 5.06±1.10% in the tumor-bearing group, compared with an increase of 4.96±1.26% in the control group. The apoptotic index (AI) of the skeletal muscle in the tumor-bearing group increased to 40.5±3.20% but was 0% in the control group, and Bax expression was strongly positive in 5 of the 10 rabbits in the tumor-bearing group, and significantly stronger than in the control group (*P*=0.0002). In the late stage of tumor bearing, 40 days after implantation, the AI had declined to 0.93±0.96% in the tumor-bearing group, but was still 0% in the control group. Bax expression was rarely detected in either the tumor-bearing group or the control group, and there was no significant difference between the two groups (*P*=0.706). No significant changes in Bcl-2 were observed in either group. The above results showed that apoptosis via Bax played a role in muscle wasting associated with progression of the malignant tumor. However, the apoptosis and expression of Bax were seen only in the early stage, within 20 days after implantation, not in the late stage. This suggested that the muscle wasting in the early stage might be caused by a different mechanism from that in the late stage.**

Key words: Apoptosis — Bax — Bcl-2 — Skeletal muscle — Cancer cachexia

Cachexia is probably the most common complication in cancer patients.[1] Anorexia, weight loss, muscle loss, atrophy, and alterations in carbohydrate, lipid, and protein metabolism can occur early in the course of cancer cachexia. Although the etiology of cancer cachexia remains unclear, it appears to be associated with metabolic competition, malnutrition, or circulating humoral factors.[2]

It is well known that muscle protein wasting occurs in the cachectic state. Increasing muscle protein degradation and decreasing muscle protein synthesis cause muscle atrophy.[3] We previously suggested that anemia-inducing substance (AIS) secreted by tumor tissue might cause cancer cachexia.[4–7] Removing AIS by plasma perfusion has been found to inhibit the increased degradation of muscle proteins in rabbits at the late stage of cachexia induced by VX2 carcinoma.[3] However, the mechanism of the muscle wasting that occurs in the early stage of tumor bearing is unknown. We previously reported observing that apoptosis occurs in normal skeletal muscle cell of VX2-bearing rabbits in the early stage after tumor implantation.[8]

Apoptosis plays a key role in various biological processes and functions and is observed in many different tumor and normal tissues. Various genes related to apoptosis have already been identified, and one of them, *bcl-2*, is known to be a suppressor of apoptosis. By contrast, another member of the *bcl-2* family, *bax*, has the function of promoting apoptosis.[9–11] The aim of this study was to investigate both the occurrence of skeletal muscle cell apoptosis in the cancer cachexia process and the association between apoptosis and the expression of Bcl-2 and Bax proteins.

## MATERIALS AND METHODS

**Experimental animals** Twenty male Japanese White rabbits weighing approximately 3 kg (SCC Co., Shizuoka) were divided into two groups, i.e., a tumor-bearing group (*n*=10), and a control group (*n*=10). The right thigh muscles of the animals in the tumor-bearing group were implanted with $1×10^5$ VX2 carcinoma cells in 1 ml of 0.15 *M* NaCl,[12] whereas the right thigh muscles of the control group were injected with 1 ml of 0.15 *M* NaCl. The animals were housed under constant temperature and humidity conditions and given access to water and food (CR-3: Clea, Tokyo) *ad libitum*. The control animals were provided with the same amount of food as the tumor-bearing group. Body weight and lean body mass (LBM) were measured every 10 days after tumor implantation.

Under general anesthesia with intravenous pentobarbital sodium (25 mg/kg), muscle tissue specimens were collected from the quadriceps muscle of the left thigh of each

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rabbit, before, and on days 10, 20, 30, and 40 after VX2 tumor implantation. The specimens were fixed with 10% neutral formalin to prepare paraffin sections or were rapidly frozen in liquid nitrogen.

**Measurement of body composition**  After anesthetizing the animals with intravenous pentobarbital sodium (25 mg/kg), their total body electrical conductivity (TOBEC) values were measured with the TOBEC Small Animal Body Composition Analysis System (inner diameter: 203 mm; 617 mm long, Model SA3203, EM-SCAN Inc., Springfield, IL). The measurements were made by observing changes in impedance in the measurement chamber when the animal was exposed to a 10-MHz magnetic field. LBM was calculated from the TOBEC value by using the formula: LBM=1.536×TOBEC value+475.1.[13]

**Transmission electron microscopy**  A 0.5 cm$^3$ specimen of muscle tissue was fixed for 24 h in 1.5% glutaraldehyde in 0.062 $M$ cacodylate buffer, pH 7.4, containing 1% sucrose and post-fixed in 1% OsO$_4$ for 2 h. The specimens were then dehydrated and embedded in resin (Polysciences, Warrington, PA). Semi-thin sections, 800 nm thick, were stained with toluidine blue for examination under a light microscope. Thin sections, 100 nm thick, were double-stained with uranyl acetate/lead citrate to examine them for ultrastructural changes with a JEM-1200 electron microscope (JOEL, Tokyo).

***In situ* detection of apoptosis**  Apoptosis in the muscle tissue sections was evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL)[14] by following the procedure of the Apoptag Plus *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD). Stained cells were counted under a light microscope. The positive controls were exposed to DNase I for 10 min at room temperature, before the TUNEL reaction. The negative controls were prepared by omitting TdT from the labeling mix. The apoptotic index (AI), defined as the ratio of TUNEL-method-positive cells in a 200-power microscopic field, was used in the analysis, and the AI values in the animal groups were compared. Two different persons blinded to the treatment counted the positive cells in three microscopic fields on one slide from each specimen, and their counts did not differ significantly.

**DNA laddering**  Skeletal muscle tissue collected from each rabbit every 10 days after tumor implantation was homogenized, and the DNA was extracted by the methods described previously.[15] The extracts were electrophoresed in 1.5% agarose gel, stained with SYBR Green I (Molecular Probes, Eugene, OR), and visualized under UV illumination.

**Immunohistochemistry**  Expression of Bax and Bcl-2 was investigated in paraffin-embedded sections by the avidin-biotin-peroxidase complex method. The 5-$\mu$m thick paraffin sections were deparaffinized and immersed in 0.3% hydrogen peroxidase to block endogenous peroxi-

dase activity. They were then subjected to an antigen retrieval procedure by immersing the slides in 10 m$M$ citrate buffer (pH 6.0) and heating in an autoclave at 121°C for 15 min. After washing in phosphate-buffered saline (PBS), tissue sections were preblocked with 10% normal goat serum for 15 min. The protocol of the DAKO LSAB2 kit Peroxidase (DAKO, Kyoto) was followed for each section. The sections were incubated overnight at 4°C with the primary antibody (Bax mouse monoclonal, Oncogene, Cambridge, MA; Bcl-2 mouse monoclonal, DAKO, in a 1:40 dilution with PBS containing 1% bovine serum albumin), and then with the second antibody (biotinylated goat anti-mouse immunoglobulin G secondary antibody, DAKO) for 1 h.[16] Next, they were incubated with streptavidin-peroxidase complex, and 3-amino-9-ethylcarbazole was used as the chromogen. The sections were counterstained with Mayer's hematoxylin. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody. The percentage of cells immunopositive for Bax and Bcl-2 was calculated by counting the cells in a 200-power field. Two investigators blinded to treatment graded Bax and Bcl-2 expression on the following scale: (−), no muscle fibers positive; (+), 1 to 25% of muscle fibers positive; (++), 26 to 50% of muscle fibers positive; (+++), 51 to 100% of muscle fibers positive.

**Western blotting**  Bax and Bcl-2 proteins in rabbit skeletal muscle were assessed by western immunoblotting. Frozen tissue was homogenized in 10 m$M$ Tris buffer (pH 7.4) containing 100 m$M$ NaCl on ice and centrifuged at 8000$g$ for 15 min. Protein concentrations in the supernatants were determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA). Then, extracts containing 20 $\mu$g of total protein were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Buckinghamshire, UK) for 1 h at 100 V. The membranes were then blocked with 5% non-fat skim milk in PBS with 0.1% Tween 20 (PBST) for 1 h at room temperature and probed overnight at 4°C with either monoclonal anti-Bax or anti-Bcl-2, the same antibody as used for the immunohistochemical analysis (1:400 dilution with PBST containing 5% non-fat skim milk). After washing with PBST, the membranes were incubated for 1 h at room temperature in PBST containing 2000-fold diluted anti-mouse IgG goat antibody conjugated with horseradish peroxidase (ICN Pharmaceuticals Inc., Aurora, OH). Finally, the membranes were developed with an enhanced chemiluminescence western blotting determination kit (ECL-Plus, Amersham Pharmacia), and Hyperfilm ECL (Amersham Life Science, Buckinghamshire, UK) was exposed to them.

**Statistics**  Student's $t$ test and Mann-Whitney's $U$ test were used to analyze the data for significant differences, and differences were considered statistically significant

when the $P$ value was <0.05. All data are expressed as means±SD.

## RESULTS

**Changes in total body weight and LBM** The total body weight of each animal was measured every 10 days after tumor implantation and expressed as a ratio to the baseline values for comparison between the two groups. At 10 days after tumor implantation the total body weight of the rabbits implanted with VX2 carcinoma had significantly decreased (to a ratio of 0.96±0.01 to their baseline weight, $P<0.01$ compared with the control group), and they continued to lose weight until 40 days after implantation (declining to a ratio of 0.879±0.026, Fig. 1A).

The LBM of each animal was also measured every 10 days and expressed as a ratio to the baseline values. LBM increased continuously in the control group (to a ratio of 1.021±0.01 on day 10 and 1.119±0.02 on day 40),

whereas in the tumor-bearing group it significantly decreased compared with the control group (to 0.975±0.01 on day 10 and 0.890±0.01 on day 40, Fig. 1B).

Table I. Changes of Apoptotic Index in the Skeletal Muscle of Tumor-bearing Rabbits

| Days after tumor implantation | Control rabbits ($n=10$) | Tumor-bearing rabbits ($n=10$) |
|---|---|---|
| 0 | 0 | 0 |
| 10 | 0 | 28.1±2.84 |
| 20 | 0 | 40.5±3.20 |
| 30 | 0 | 9.67±2.22 |
| 40 | 0 | 0.93±0.96 |

The apoptotic index was calculated based on the proportion of TUNEL-positive cells detected at a magnification of ×200. The average values obtained from 10 animals for each group are shown.
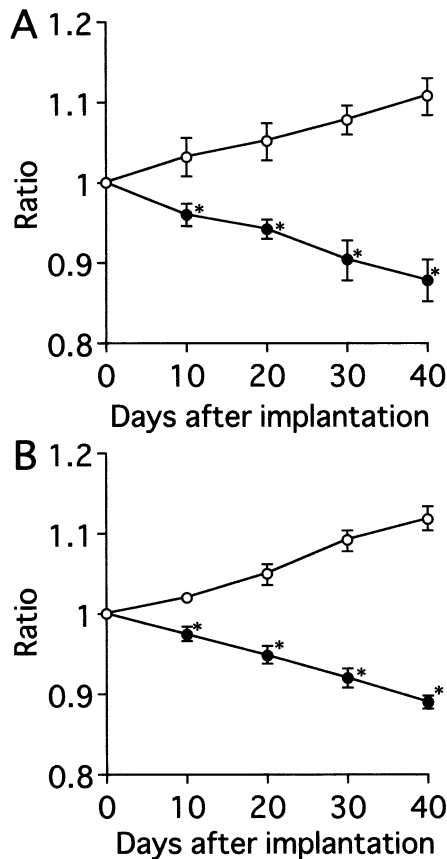Values are expressed as means±SD.



Fig. 1. Total body weight (A) and lean body mass (B) of VX2-carcinoma-bearing and control rabbits. Closed circles, VX2 implanted rabbits ($n=10$); open circles, control rabbits ($n=10$). Values are means±SD expressed as ratios to the value on day 0. Significance: ∗, $P<0.05$ vs. control group (Student's $t$ test).
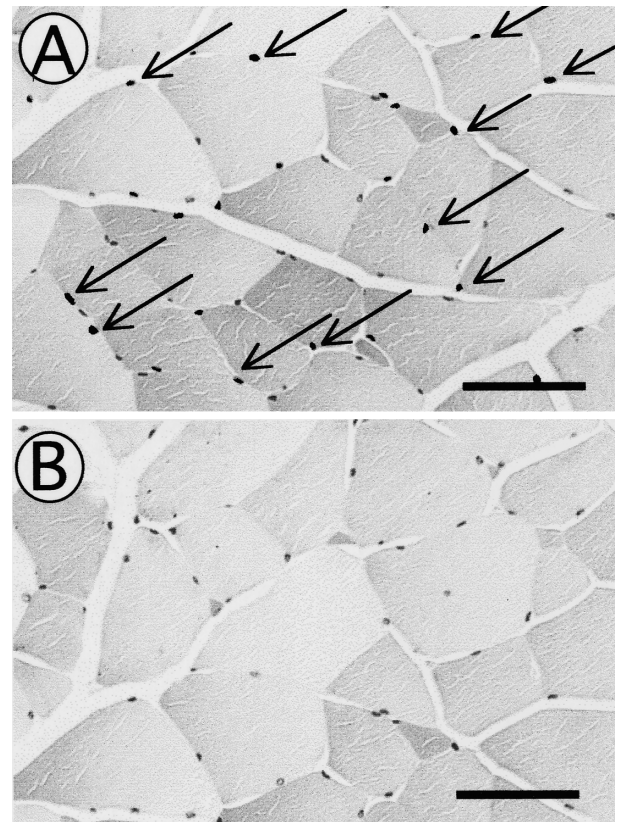


Fig. 2. TUNEL signals in the skeletal muscle cells of a VX2-bearing and control rabbit. (A) TUNEL signals were demonstrated in the nuclei of some skeletal muscle cells in VX2-bearing rabbit on day 20 after tumor implantation with AI of 40.5±3.20% (arrows). (B) Skeletal muscle cells of a control rabbit on day 20: no stained nuclei are visible. Scale bars, 30 $\mu$m.

**AI** Examination of muscle cell apoptosis in the skeletal muscle tissue collected at 10-day intervals after tumor implantation revealed an AI value in the tumor-bearing group of 28.1±2.84 on day 10 (Table I). By day 20, many apoptotic cells were found (AI: 40.5±3.20, Fig. 2A), but the number of apoptotic cells gradually decreased after day 20 (AI: 9.67±2.22 on day 30 and 0.93±0.96 on day 40, Table I). By contrast, no apoptotic cells were detected in the control group at any of the times examined (Fig. 2B).

**Ultrastructural changes in muscle cells** Muscle cell apoptosis was confirmed by electron microscopy. The muscle cells in the tumor-bearing group exhibited the characteristic features of apoptosis, i.e., cytoplasmic shrinkage and nuclear chromatin condensation (Fig. 3A), but no morphological changes were detected in the control group (Fig. 3B).

**DNA laddering pattern** A DNA laddering pattern characteristic of DNA fragmentation was visible in the muscle tissue of the tumor-bearing group, but not in the control group. The ladder phenomenon was much more evident in the samples from animals with higher percentages of TUNEL-positive nuclei (Fig. 4).

**Expression of Bax and Bcl-2 proteins** Immunostaining specific for Bax and Bcl-2 in skeletal muscle tissue is shown in Table II, Fig. 5. Bcl-2 immunoreactivity was either not detected at all or only weakly observed in both groups. No trends in Bcl-2 expression were perceived in either group throughout the study period. By contrast, Bax expression increased gradually after tumor implantation in the tumor-bearing group (Table II). By 10 days after tumor
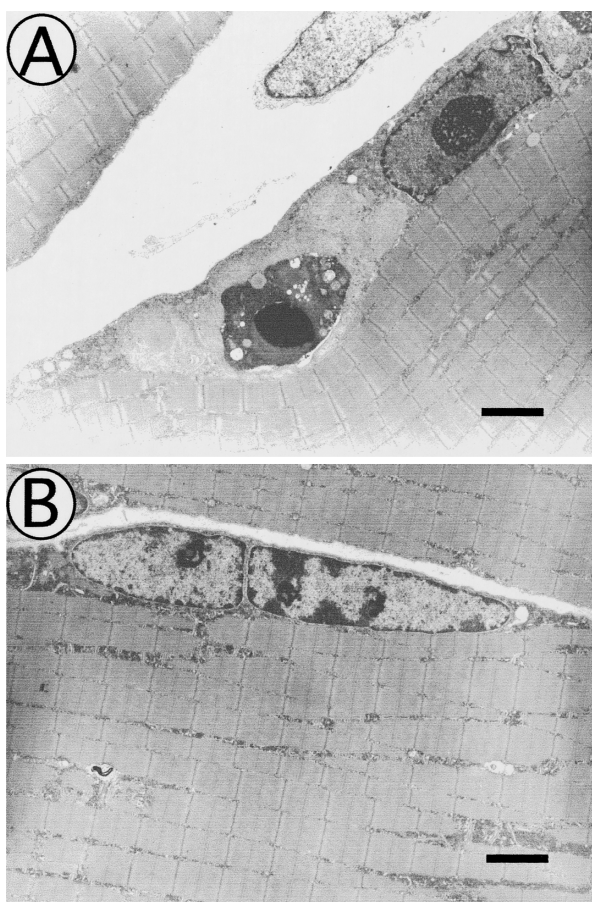
Fig. 3. Muscle cell apoptosis in VX2-bearing rabbits. Electron micrographs of muscle tissue from a tumor-bearing rabbit 20 days after tumor implantation (A) and a normal control animal on day 20 (B). A, B ×8000. Scale bars, 1 μm.
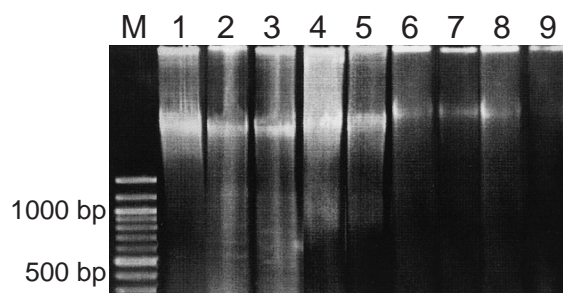
Fig. 4. DNA fragmentation assay of rabbit skeletal muscle. Lane M: DNA ladder marker. Lane 1: tumor bearing, day 0. Lane 2: tumor bearing, day 10. Lane 3: tumor bearing, day 20. Lane 4: tumor bearing, day 30. Lane 5: tumor bearing, day 40. Lane 6: control, day 10. Lane 7: control, day 20. Lane 8: control, day 30. Lane 9: control, day 40.

Table II. Expression of Bcl-2 and Bax in the Skeletal Muscle of Tumor-bearing Rabbits

| Days after tumor implantation | | Control (n=10) (−/+/++/+++) | Tumor-bearing (n=10) (−/+/++/+++) | P value |
|---|---|---|---|---|
| 0 | Bax | 9/1/0/0 | 8/2/0/0 | 0.706 |
| | Bcl-2 | 9/1/0/0 | 10/0/0/0 | 0.706 |
| 10 | Bax | 10/0/0/0 | 1/5/4/0 | 0.0007 |
| | Bcl-2 | 10/0/0/0 | 9/1/0/0 | 0.706 |
| 20 | Bax | 9/1/0/0 | 0/3/2/5 | 0.0002 |
| | Bcl-2 | 9/1/0/0 | 10/0/0/0 | 0.706 |
| 30 | Bax | 8/2/0/0 | 4/5/1/0 | 0.054 |
| | Bcl-2 | 8/2/0/0 | 8/2/0/0 | >0.99 |
| 40 | Bax | 10/0/0/0 | 9/1/0/0 | 0.706 |
| | Bcl-2 | 10/0/0/0 | 9/1/0/0 | 0.706 |

Bcl-2 and Bax positivity evaluated as follows: (−), lack of positivity; (+), 1–25% positive muscle cells; (++), 26–50% positive muscle cells; (+++), 51–100% positive muscle cells.
Statistical analysis was performed with Mann-Whitney's U test.

implantation, Bax expression in skeletal muscle cell was graded "++" in 4 of the 10 tumor-bearing rabbits, and by day 20, it was graded "+++" in 5 of the 10 tumor-bearing
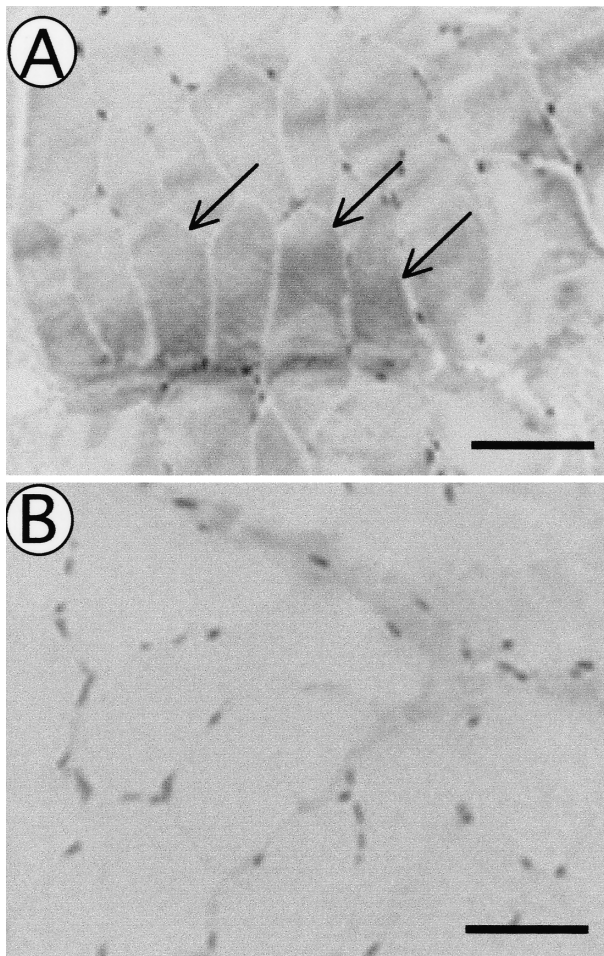


Fig. 5. Immunostaining for Bax and Bcl-2 proteins in rabbit skeletal muscle cells. (A) Increased Bax immunoreactivity (arrows) is observed in the cytoplasm of skeletal muscle cells of a tumor-bearing rabbit 20 days after tumor implantation. (B) No Bax immunoreactivity was observed in a control rabbit on day 20. Scale bars, 30 $\mu$m.
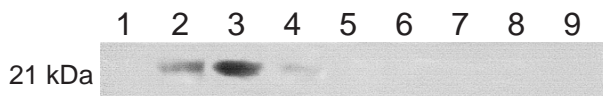


Fig. 6. Western blot analysis of Bax extracted from rabbit skeletal muscle. Lane 1: tumor bearing, day 0. Lane 2: tumor bearing, day 10. Lane 3: tumor bearing, day 20. Lane 4: tumor bearing, day 30. Lane 5: tumor bearing, day 40. Lane 6: control, day 10. Lane 7: control, day 20. Lane 8: control, day 30. Lane 9: control, day 40.

rabbits. By contrast, hardly any Bax-immunopositive cells were detected in the control group (Mann-Whitney's $U$ test, tumor-bearing vs. control, $P=0.0007$ on day 10, $P=0.0002$ on day 20, Table II). After day 20, however, Bax immunoreactivity decreased continuously in the tumor-bearing group. By 40 days after tumor implantation, Bax immunopositivity was "+" in only one tumor-bearing rabbit, and no significant difference was found between the two groups ($P=0.706$, Table II).

The results of the immunohistochemical analysis were further confirmed by the results of the western blot analysis (Fig. 6). Bax protein expression was significantly enhanced in the tumor-bearing rabbits, especially on day 20 after tumor implantation.

## DISCUSSION

Cancer cachexia is accompanied by progressive body weight loss, anorexia, anemia, and immunodeficiency.[1] There have been reports that cancer cachexia is caused by cytokines, such as tumor necrosis factor (TNF)-α,[17, 18] interleukin (IL)-6,[19] and interferon (IFN)-γ,[20] that are secreted by tumor cells or host cells. We previously identified AIS in plasma collected from cancer cachexia patients and later discovered that AIS produces anemia and immunodeficiency and suppresses the growth of adipocytes and skeletal muscle cells.[4–7, 21] Todorov et al.[22] discovered that proteolysis-inducing factor (PIF) is produced by MAC16 tumor and reported that PIF contributes to muscle wasting. The cause of cancer cachexia, however, remains to be clarified.[23]

We studied cancer cachexia by using VX2-tumor-bearing rabbits and found that body weight loss, anemia, and immunodeficiency became marked at 30 to 40 days after implantation.[24] In the experiments in rats and mice reported thus far, weight loss was mainly examined in the stage in which the cachexia is obvious.[25] We, however, developed an original system for measuring LBM in rabbits and have used it to chart the time course of body weight and LBM changes beginning with the early stage after implantation. The results showed that LBM and body fat began to decrease in the early stage after implantation, when the pathological manifestations of cachexia were still not marked. We have also reported the occurrence of apoptosis of normal skeletal muscle in the early stage of tumor bearing,[8] and in the present study we investigated its pathogenetic mechanism.

Apoptosis, programmed cell death, is a part of the normal process of development and differentiation in multicellular organisms, and it also plays an important role in the cell death of normal cells and tumor cells.[26–28] The Bcl-2 family is highly involved in the control of apoptosis, and Bcl-2 protein is present in the mitochondrial outer membrane and the nuclear membrane.[29] Overexpression

of Bcl-2 suppresses apoptosis,[10] though one of the homologues of Bcl-2 that have been found, Bax, promotes apoptosis. It is claimed that Bcl-2 and Bax form homodimers and heterodimers, and that the ratio between them controls apoptosis.[10, 11] This apoptosis path via the Bcl-2 family has been noted in many other diseases, and some reports state that it participates in the atrophy of normal skeletal muscle in artificially induced chronic heart failure in rats.[30, 31] However, there have been no reports of the Bcl-2 family being associated with skeletal muscle atrophy in cancer cachexia.

The two groups in the present study were examined for the presence of apoptosis in skeletal muscle by electron-microscopic observation of morphology, staining by the TUNEL method, and by the DNA ladder method. The results showed that apoptosis was first noticed 10 days after implantation in the tumor-bearing group. The apoptosis in skeletal muscle peaked at 20 days after implantation, declined thereafter, and was hardly seen at all by 40 days after implantation. In addition, expression of Bax and Bcl-2 was examined by immunohistochemical analysis and western blotting. The results showed that expression of Bax increased in the tumor-bearing group and that it coincided with the occurrence of apoptosis. However, no significant changes were noted in the expression of Bcl-2. Apoptosis was rarely observed in the control group. The above findings strongly suggest that Bax is related to apoptosis in skeletal muscle in the early stage of tumor bearing.

Apoptosis of skeletal muscle cells tends to decrease in the late stage after implantation, but in cancer cachexia muscle wasting continues until the individual dies, and thus the muscle wasting in the late stage of tumor bearing is probably affected by factors other than those that cause apoptosis. We investigated DNA synthesis in skeletal muscle cells after tumor implantation in VX2-tumor-bearing rabbits and discovered that it was markedly reduced in the late stage after implantation. Plasma perfusion with a non-coated charcoal filter, however, restored DNA synthesis to normal.[3] In addition, plasma perfusion improved muscle wasting in the late stage of tumor bearing, even though no change was noted in apoptosis in the early stage.[32]

As mentioned above, there are probably large differences between the mechanism of the muscle wasting associated with the progression of malignant tumor in the early and late stage of tumor bearing. The main difference seems to be the occurrence of apoptosis in the early stage and metabolic changes induced by humoral factors in the late stage. This study is the first to demonstrate that the muscle wasting via apoptosis in the early stage of tumor bearing may be controlled by Bax, an apoptosis-related protein.

Apoptosis is regulated by the balance of anti-apoptotic factors, such as Bcl-2 and Bcl-$X_L$, and pro-apoptotic factors, such as Bax and Bak. Fas and p53 also play important roles in the regulation of apoptosis. The mechanisms of implication of these components in muscle apoptosis of cancer cachectic subjects need to be elucidated.

The daily life of cancer patients is highly restricted by the skeletal muscle atrophy that results from cancer cachexia, causing a severe reduction in quality of life (QOL). However, no effective therapy has yet been established. Although many factors may be associated with the muscle wasting in cancer cachexia, the results of the present study strongly suggest involvement of Bax, an apoptosis-related protein. Clarification of the mechanism of apoptosis in cancer cachexia may lead to a gene therapy that would prevent tumor-induced weight loss.

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